ORIGINAL ARTICLE

Janus-kinase-2 relates directly to portal hypertension and to complications in rodent and human cirrhosis

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ABSTRACT

Objective Angiotensin II (AngII) activates via angiotensin-II-type-I receptor (AT1R) Janus-kinase-2 (JAK2)/Arhgef1 pathway and subsequently RHOA/Rho-kinase (ROCK), which induces experimental and probably human liver fibrosis. This study investigated the relationship of JAK2 to experimental and human portal hypertension.

Design The mRNA and protein levels of JAK2/ARHGEF1 signalling components were analysed in 49 human liver samples and correlated with clinical parameters of portal hypertension in these patients. Correspondingly, liver fibrosis (bile duct ligation (BDL), carbon tetrachloride (CCl_4)) was induced in floxed-*Jak2* knock-out mice with SM22-promotor (SM22^{Cre+}-*Jak2*$^{f/f}$). Transcription and contraction of primary myofibroblasts from healthy and fibrotic mice and rats were analysed. In two different cirrhosis models (BDL, CCl_4) in rats, the acute haemodynamic effect of the JAK2 inhibitor AG490 was assessed using microsphere technique and isolated liver perfusion experiments.

Results Hepatic transcription of JAK2/ARHGEF1 pathway components was upregulated in liver cirrhosis dependent on aetiology, severity and complications of human liver cirrhosis (Model for End-stage Liver disease (MELD) score, Child score as well as ascites, high-risk varices, spontaneous bacterial peritonitis). SM22^{Cre+}-*Jak2*$^{f/f}$ mice lacking *Jak2* developed less fibrosis and lower portal pressure (PP) than SM22^{Cre-}-*Jak2*$^{f/f}$ upon fibrosis induction. Myofibroblasts from SM22^{Cre+}-*Jak2*$^{f/f}$ mice expressed less collagen and profibrotic markers upon activation. AG490 relaxed activated hepatic stellate cells in vitro. In cirrhotic rats, AG490 decreased hepatic vascular resistance and consequently the PP in vivo and in situ.

Conclusions Hepatic JAK2/ARHGEF1/ROCK expression is associated with portal hypertension and decompensation in human cirrhosis. The deletion of *Jak2* in myofibroblasts attenuated experimental fibrosis and acute inhibition of JAK2 decreased PP. Thus, JAK2 inhibitors, already in clinical use for other indications, might be a new approach to treat cirrhosis with portal hypertension.

Significance of this study

What is already known on this subject?

- Angiotensin-II-type-I receptor (AT1R) stimulation activates RHOA/Rho-kinase (ROCK) via Janus-kinase-2 (JAK2) in extrahepatic vessels.
- AT1R/JAK2/ROCK is involved in the development of liver fibrosis.
- AT1R/JAK2/ROCK is upregulated in human and animal fibrosis compared with non-fibrotic hepatic tissue, especially in activated hepatic stellate cells (HSCs).
- JAK2 inhibition blunts fibrogenesis in animal models of fibrosis.

What are the new findings?

- Hepatic expression of renin–angiotensin system components and JAK2/ROCK pathway are strongly correlated with each other in human cirrhosis.
- Activation of AT1R/JAK2/ROCK pathway increases with severity of liver cirrhosis and portal hypertensive complications in humans.
- Myofibroblast-specific deletion of JAK2 blunts fibrogenesis and decreases portal pressure (PP) in two different mice models of fibrosis.
- Acute JAK2 inhibition relaxes activated HSCs and decreases PP in two different rat models of liver cirrhosis.

How might it impact on clinical practice in the foreseeable future?

- JAK2 inhibitors are already in clinical use for other indications.
- JAK2 inhibitors may provide a novel therapy concept to reduce liver fibrosis and decrease PP.

INTRODUCTION

Chronic liver diseases impose a major burden in health systems.[1,2] Although of different aetiologies, they share a common end stage, namely liver cirrhosis with portal hypertension.[3,4] Increased hepatic vascular resistance due to progressive hepatic fibrosis and active contraction of myofibroblasts characterises liver cirrhosis with portal hypertension.[5] The main sources of these myofibroblasts are activated hepatic stellate cells (HSCs).[6] They express α-smooth muscle actin (αSMA) as a marker of activation.[5,6]

One of the main drivers of HSC activation causing fibrogenesis and the development of portal hypertension is the renin–angiotensin system (RAS) via stimulation of the angiotensin-II-type-I receptor (AT1R)[7–10] (figure 1). Recently, it has been shown that AT1R is coupled to the tyrosine kinase Janus-kinase-2 (JAK2), which in turn activates the RhoA/Rho-kinase (ROCK) pathway (figure 1).[10 11] Furthermore, we recently provided evidence that in activated HSCs, the AT1R stimulation phosphorylates JAK2, which in turn induces Arhgef1, the nucleotide exchange factor responsible for activation of RhoA, which subsequently activates ROCK, crucially involved in fibrosis and portal hypertension.[10–17] As a consequence, JAK2 inhibition decreased fibrosis in animal models.[10] These data were confirmed by another group using long-term inhibition of JAK2. This study linked the transcription regulators of the Signal Transducers and Activators of Transcription (STAT) family to liver fibrosis.[18]

While the long-term treatment with JAK2 inhibitors decreases experimental fibrosis,[10 18] the acute effect of JAK2 inhibition on portal pressure (PP) remains unknown. Acute modulation of PP is of important clinical relevance.

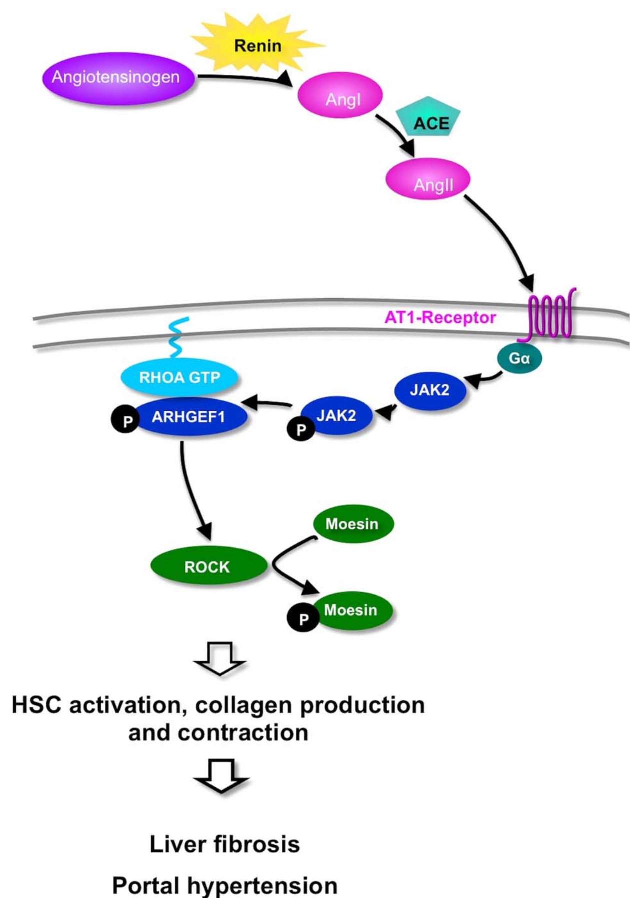

Figure 1 Schematic illustration of the renin–angiotensin system and the downstream components of the angiotensin-II-type-I receptor (AT1R). Renin cleaves angiotensin I (AngI) from angiotensinogen, which is further transformed to angiotensin II (AngII) by ACE. Binding of AngII to its receptor AT1R induces the activation of Janus-kinase-2 (JAK2) and thereby the phosphorylation of ARHGEF1. ARHGEF1 activates RHOA, which in turn activates Rho-kinase (ROCK). The activity of ROCK can be measured by the phosphorylation of its substrate Moesin. Activation of AT1R/JAK2/ROCK pathway leads to activation of hepatic stellate cells (HSCs) and fibrosis,[10] but also to increased contraction of activated HSCs and ultimately to portal hypertension in cirrhosis as outlined below.

The present study assessed a relationship between decompensation of liver cirrhosis and activation of JAK2/ROCK pathway. Additionally, using different experimental models of cirrhosis, the effect of myofibroblast-specific *Jak2* deficiency on fibrogenesis and portal hypertension was examined. Furthermore, we evaluated the effect of acute JAK2 inhibition on relaxation of activated HSCs and PP.

MATERIALS AND METHODS

Human samples

The human ethics committee of the University of Bonn (029/13) approved the use of human samples. Liver samples and hepatic arteries were taken at liver transplantation between 1999 and 2005 (n=49) and samples from non-cirrhotic samples served as controls (n=10).[10 13 19 20]

Quantitative real time-PCR

mRNA levels were measured, as described previously.[10 12 13] Assays provided by Applied Biosystems (Foster City, USA) are listed in online supplementary table S1. *18S* rRNA served as endogenous control. The results of HSCs and liver samples were expressed as $2^{-\Delta\Delta Ct}$, and express the x-fold increase of gene expression compared with the control group.

Coimmunofluorescence stainings

Colocalisation of either αSMA or CD68 with ROCK and pMoesin was analysed by immunofluorescent staining of 7 mm cryosections, as described previously.[10] Briefly, the sections were incubated overnight with a primary antibody mix of mouse-anti-SMA (αSMA antibody A2547; Sigma-Aldrich, Munich, Germany) or mouse-anti-CD68 (CD68 antibody ab955; Abcam, Cambridge, UK) and rabbit-anti-ROCK (ROCK2 antibody sc5561; Santa Cruz, Dallas, USA) or rabbit-anti-pMoesin (pMoesin antibody sc12 895; Santa Cruz) diluted each 1:100 in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). Thereafter, incubation with secondary antibodies was performed in darkness. Secondary antibodies were diluted 1:300 in 1% BSA/PBS (GαM Alexa Fluor 488 IgG and GαR Alexa Fluor 594 IgG; Life technologies, Eugene, USA) and cryosections were incubated for 2 h, following incubation with 4′,6-diamidino-2-phenylindole (DAPI) (1:10 000; Sigma-Aldrich). Finally, sections were covered with Fluorescent Mounting Medium (S3023; Dako, Carpinteria, USA). Analyses were performed with confocal laser scanning microscopy (LSM-710; Carl Zeiss, Jena, Germany) and evaluated using Zen lite (black edition; Carl Zeiss).

Western blotting

Snap-frozen cells and liver samples were processed, as previously described.[10 12 13] Ponceau-S staining assured equal protein loading. Glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) or β-actin served as endogenous controls. Membranes were incubated with the respective primary antibody (see online supplementary table S2) and corresponding secondary peroxidase-coupled antibody (Santa-Cruz-Biotechnology, Santa Cruz, California, USA). After enhanced chemiluminescence (ECL, Amersham, UK), digital detection was evaluated using Chemi-Smart (PeqLab, Biotechnologies, Erlangen, Germany).

ELISA for pJAK2

The ELISA assay to measure pJAK2 in human liver samples was provided by RayBiotech, Inc. (RayBio phospho-JAK2 ELISA Kit; Norcoss, Georgia, USA).

pJAK2 was measured in human liver samples by ELISA according to the ELISA Kit Protocol. In brief, human liver samples were homogenised and diluted to 0.1 μg/μL. One hundred microlitres of each sample was used for each well and incubated with 1× biotinylated anti-phosphotyrosine antibody. After further incubation with 1× horseradish peroxidase (HRP)–Streptavidin solution and with stop solution, the pJAK2 content was measured at 450 nm. The measured values are compared with the values from the healthy livers, which are set as 100 arbitrary units.

Animals

We used 80 Sprague–Dawley wild type (WT) rats and 179 mice (75 C57BL/6J wild type (wt), 104 SM22^{Cre}-*Jak2*$^{f/f}$ mice) for our experiments (see online supplementary table S3). SM22^{Cre}-*Jak2*$^{f/f}$ mice breeding pairs were kindly provided by Peter Sayeski, Department of Physiology and Functional Genomics, University of Florida, College of Medicine, Gainesville, Florida, USA. The responsible committee for animal studies in North Rhine-Westphalia approved the study (LANUV 84-02.04.2014.A137).

Cholestatic model of fibrosis

Bile duct ligation (BDL) was performed in rats, as described previously.[12 13] BDL and sham operation were performed in 20–25 g mice, which were sacrificed after 2 weeks. Haemodynamic experiments were carried out 4–5 weeks after BDL in 19 rats, when rats developed ascites. Twelve sham-operated rats served as controls.

Toxic model of fibrosis

Periodical carbon tetrachloride (CCl_4) inhalation of 2 L/min was performed in 47 mice (15 C57BL/6J wt, 32 SM22^{Cre}-*Jak2*$^{f/f}$ mice) for 4 weeks, as described previously,[10 21] while 56 mice served as controls (24 C57BL/6J wt, 32 SM22^{Cre}-*Jak2*$^{f/f}$ mice). Nineteen rats with an initial body weight of 100–120 g underwent twice weekly inhalation of 1 L/min CCl_4 for 14–16 weeks until ascites was present, as described previously.[12 13 22] Twenty four age-matched control rats did not receive CCl_4.

Hepatic hydroxyproline content

In analogue segments (200 mg) of snap-frozen livers, the hepatic hydroxyproline content was determined photometrically, as described previously.[10 14]

Isolation of primary HSCs and myofibroblasts

Rat HSCs and mouse myofibroblasts were isolated, as described previously.[10 13] Briefly, primary myofibroblasts and HSCs were isolated in a two-step pronase–collagenase perfusion from the livers of healthy and fibrotic (2 weeks after BDL) rats (n=6), as well as wt (n=36) or SM22^{Cre}-Jak2$^{f/f}$ mice (n=40) and fractionated by density gradient centrifugation. Viability and purity were systematically over 95%. Cells were seeded on uncoated plastic culture dishes. Experiments were performed 7 days after isolation or after the first passage (10 days) when myofibroblasts and HSCs were fully activated.

Incubation with angiotensin II and AG490

Angiotensin II (AngII) (5 μM) and/or AG490 (1.5 μM) were added to the culture medium of these cells as indicated for 3 days, or cells remained untreated, as previously described.

3D stress-relaxed collagen lattice contraction model

The ability of HSCs to contract 3D collagen matrices was measured in hydrated collagen gels, as previously described.[12 13] AG490 was added in different concentrations (0.05; 0.5; 5; 25 μM). Control cell-free gels provided estimates for the pre-contraction volume and determination of relative changes in volume (% contraction). All data are from experiments using at least three sets of three collagen lattices with culture-activated HSCs from three different rat HSC isolations.

In situ liver perfusion

In situ liver perfusion was performed in a recirculating system in 10 cirrhotic CCl_4 rats and 14 control rats, as described previously.[12 13] The criteria for liver viability included gross appearance of the liver, stable perfusion, bile production >0.4 μL/min*g (except BDL rats) and stable buffer pH (7.4±0.1) during the initial 20 min stabilisation period. If one of the viability criteria was not met, the experiment was discarded.

Effect of the JAK2 inhibitor on hepatic vascular resistance

Livers were initially perfused at a constant flow (30 mL/min) for a period of 20 min without any interference in order to stabilise the entire system. The AT1R agonist AngII (5 μM) or the α_1-adrenergic receptor agonist methoxamine (200 μM) was applied to the perfusate to increase hepatic vascular resistance. Then, cumulative concentration–response curves with AG490 (10^{-6}–10^{-3} M) were obtained by adding the JAK2 inhibitor or solvent into the perfusate every 10 min. Data are expressed as percentage of the basal perfusion pressure.

Role of AT1R blocker in the AG490-mediated effect

In order to investigate the AT1R dependency, Losartan (10 mg) was added into the perfusate prior to AG490 in CCl_4 rats, as previously described.[12 13]

Haemodynamic studies

The rats used for haemodynamic studies showed typical features of portal hypertension, including increased spleen weight (healthy 0.76 g±0.04 g vs cirrhotic 1.24 g±0.14 g, $p<0.05$). Haemodynamic studies were performed under ketamine/xylacine anaesthesia (78 mg/kg/12.5 mg/kg intramuscularly), as previously described.[12 13 23 24] *Microsphere technique*. Cardiac output was measured using the coloured microsphere method, as previously described.[12 13 23 24] Briefly, after stabilisation, PP and mean arterial pressure (MAP) were monitored for 20 min, followed by application of microsphere technique. Then, JAK2 inhibitor, AG490 (1 mg/kg), was administered intravenously and PP and MAP were monitored for further 60 min, followed by application of the microsphere technique.

Analysis of aortic ring contraction

Aortic ring contraction was performed, as described previously.[20] In brief, freshly excised aortas from CCl_4-intoxicated rats were cut into 3–4 mm wide rings and mounted in organ bath chambers, as previously described.[19] Aortic rings were pre-contracted with AngII (5 μM) and the effects of JAK2 inhibition by AG490 on aortic ring contraction were assessed dose-dependently (10^{-6}M–10^{-3} M).

Statistical analysis

Data are presented as means±SD. Mann–Whitney U test was used for comparison between groups. Wilcoxon test was used for paired comparison in the same group. Spearman rank was used for correlations of human data and the coefficient (Rs) was shown. p Values <0.05 were considered statistically significant.

RESULTS

Hepatic transcripts from RAS and *JAK2/ROCK* pathway correlate with each other, severity of liver disease and complications of portal hypertension in humans

The transcription levels of the RAS and *JAK2/ROCK* pathway components were analysed in the human situation. The general characteristics of these patients are summarised in table 1.

In these samples, the hepatic transcription of RAS components, especially *ACE* and *AT1R*, was strongly correlated with components of the *JAK2/ROCK* pathway (table 2). Furthermore, hepatic transcription of *JAK2*, *ARHGEF1*, *RHOA* and *ROCK* was strongly correlated with each other (figure 2A–D).

Since JAK2 expression and phosphorylation were shown to be located in αSMA-positive cells,[10] we analysed ROCK expression and activity downstream of JAK2. In human cirrhotic liver tissue, ROCK expression and activity, as shown by pMoesin, was colocalised to αSMA-positive cells, but not to CD68-positive macrophages (eg, Kupffer cells) (figure 2E, see online supplementary figure S1c).

JAK2/ROCK pathway is upregulated and overactivated in patients with more severe liver disease and complications of portal hypertension

Previously, we described that the components of the AT1R/JAK2 pathway (AT1R, JAK2, pJAK2, ARHGEF1, RHOA, ROCK, pMoesin) were highly expressed and activated in livers of cirrhotic patients compared with controls and that pJAK2 was mainly located in activated HSCs in these livers.[10]

Interestingly, hepatic *JAK2* transcription is significantly increased in patients with MELD above 15 or Child C cirrhosis (figure 3A). Patients with Child–Pugh score C showed significantly higher protein expression of the components of AT1R/JAK2/ROCK pathway than patients with less decompensation (figure 3B, C). Furthermore, in patients with higher MELD score or Child–Pugh score C, the hepatic activation of JAK2, investigated by phosphorylation of JAK2 at Tyr1007/1008 using ELISA, was significantly increased (figure 3D). Downstream of JAK2, the hepatic activity of ROCK, investigated by phosphorylation of its substrate Moesin at Thr558 using western blot, was significantly higher in patients with Child–Pugh score C than in patients with Child–Pugh score B (figure 3B, C). Besides the severity of liver disease, also complications of portal hypertension were associated with upregulation of the JAK2/ROCK pathway. Hepatic transcription of *JAK2* was significantly more increased in patients with ascites, high-risk oesophageal varices or spontaneous bacterial peritonitis (SBP) than in those without (figure 3E). Interestingly, in patients with ascites, as an unequivocal indicator for the presence of portal hypertension, the protein expression of the components of AT1R/JAK2/ROCK pathway was significantly higher than in patients without ascites (figure 3F, G). Furthermore, also JAK2 activation was significantly higher in patients with ascites, high-risk oesophageal varices and SBP (figure 3H, I). Additionally, signalling downstream of JAK2 was increased in patients with complications of liver cirrhosis, as shown by increased ROCK activity in patients with ascites (figure 3F, G).

Patients suffering from alcoholic liver disease showed higher levels of JAK2 expression and activation when compared with patients with non-alcoholic liver disease (including chronic viral hepatitis, see online supplementary figure S1a, b).

Table 1 General characteristics of patients included for the hepatic transcription and protein expression of the components of the renin–angiotensin system and AT1R/JAK2 pathway

Parameters	Value
Patients (n)	49
Gender (female/male)	19/30
Age (in years) as median (range)	46 (24–64)
Aetiology (alcoholic/chronic hepatitis)	13/36
MELD score as median (range)	15 (6–32)
Child score as median (range)	9 (5–12)
Child category (A/B/C)	5/24/20
Ascites (absent/mild/severe)	17/18/14
Hepatic encephalopathy (absent/present)	32/17
History of hepatorenal syndrome (no/yes)	41/8
Oesophageal varices (absent/grade I–II/grade III–IV)	7/34/8
History of variceal bleeding (no/yes)	30/19

AT1R, angiotensin-II-type-I receptor; JAK2, Janus-kinase-2.

Deficiency of *Jak2* in myofibroblasts attenuates fibrosis and decreases PP by downregulation of *Arhgef1/RhoA/Rock* pathway

Next, we evaluated the functional involvement of activated *Jak2* in various murine fibrosis models. Therefore, we used floxed-*Jak2* knock-out mice with SM22-promotor ($SM22^{Cre+}$-$Jak2^{f/f}$). αSMA-positive cells have an *Jak2* deficiency. Interestingly, myofibroblasts isolated from $SM22^{Cre+}$-$Jak2^{f/f}$. In these mice showed significantly lower transcription of *Jak2/Arhgef1/Rock* pathway compared with HSCs from $SM22^{Cre-}$-$Jak2^{f/f}$ (figure 4A). Moreover, even though *αSma* expression, as marker of myofibroblast activation, was only slightly decreased, collagen transcription was significantly blunted in HSCs from $SM22^{Cre+}$-$Jak2^{f/f}$ mice (figure 4A).

Interestingly, $SM22^{Cre+}$-$Jak2^{f/f}$ mice showed less hepatic fibrosis, induced by two different models (BDL, CCl_4), compared with fibrotic $SM22^{Cre-}$-$Jak2^{f/f}$, as shown by the lower hydroxyproline levels (figure 4B). This was accompanied by lower PP in $SM22^{Cre+}$-$Jak2^{f/f}$ (figure 4C).

Since it has been shown that primary HSCs isolated from fibrotic animals show a phenotype different from healthy animals, primary HSCs isolated from BDL rats were isolated to confirm our results (figure 4D). In these HSCs, AngII induced increased transcription of *αSma* and *Col1a1* (collagen type I), as well as upregulation of *Jak2/Arhgef1/Rock* pathway (figure 4D). JAK2 inhibition by AG490 blunted these effects markedly (figure 4D), confirming the pivotal role of JAK2 in the activation of HSCs and hepatic fibrosis.[10]

Table 2 Correlations of hepatic transcriptional levels of the components of the renin–angiotensin system and *JAK2/ROCK* pathway in specimen of homogenised liver tissues from the cirrhotic patients described in table 1

	Angiotensinogen	Renin	ACE	AT1
JAK2	r_s=0.512 p<0.0001	r_s=0.542 p<0.0001	r_s=0.856 p<0.0001	r_s=0.764 p<0.0001
ARHGEF1	r_s=0.370 p=0.016	r_s=0.395 p=0.012	r_s=0.866 p<0.0001	r_s=0.853 p<0.0001
RHOA	r_s=0.455 p=0.002	r_s=0.387 p=0.014	r_s=0.924 p<0.0001	r_s=0.925 p<0.0001
ROCK	rs=0.460 p=0.002	rs=0.344 p=0.030	rs=0.900 p<0.0001	rs=0.871 p<0.0001

AT1, angiotensin-II-type-I; *JAK2, Janus-kinase-2*; *ROCK, RhoA/Rho-kinase*.

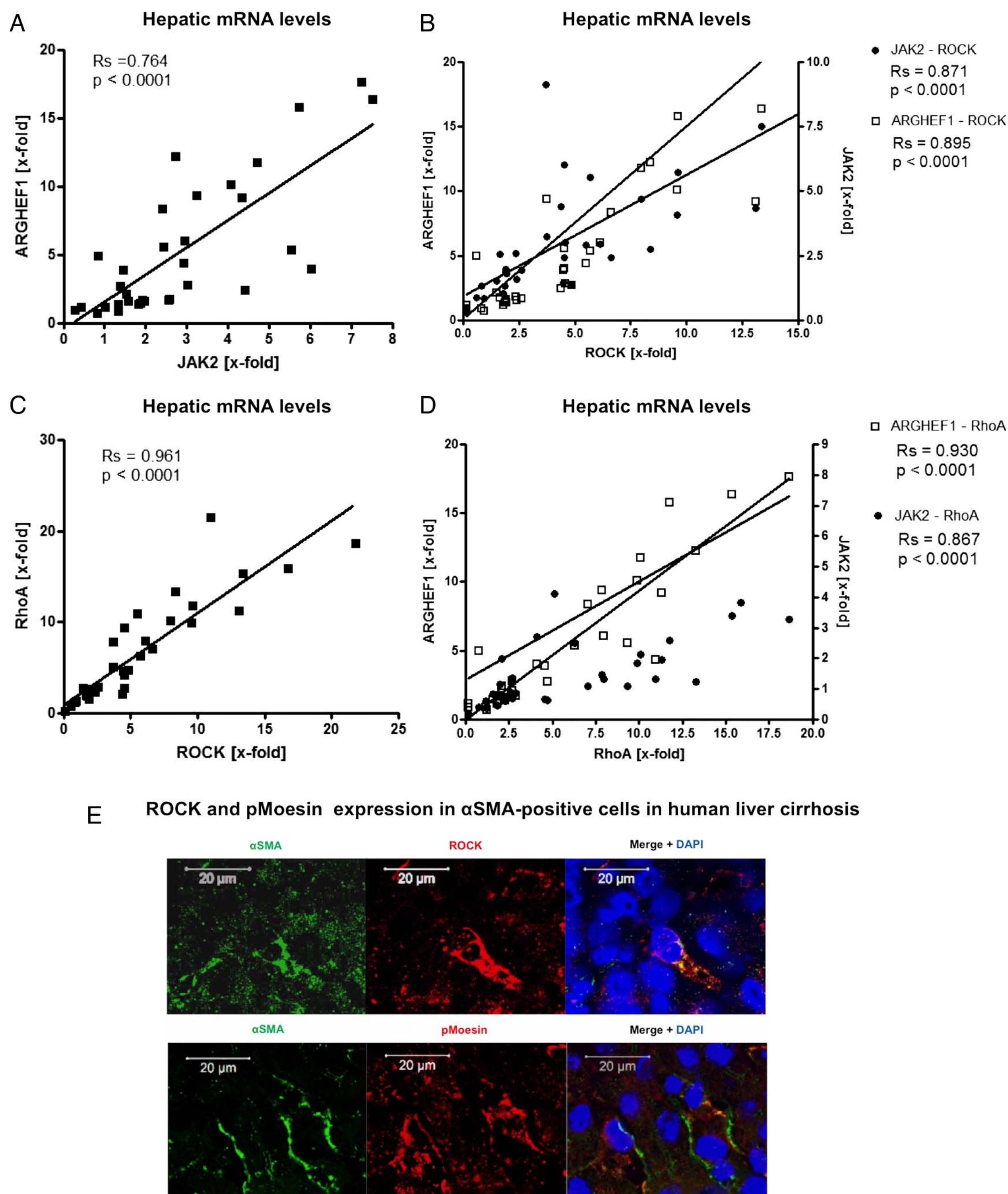

Figure 2 Hepatic transcripts from *Janus-kinase-2* (JAK2)*/RHOA/Rho-kinase* (*ROCK*) pathway correlate with each other, severity of liver disease and complications of portal hypertension in humans. The hepatic mRNA levels of *JAK2*, *ARHGEF1*, *RHOA* and *ROCK* were measured in 49 cirrhotic patients using quantitative real time-PCR. The mRNA levels of *ARHGEF1* with *JAK2* (A); *ARHGEF1* (□) and *JAK2* (●) with *ROCK* (B); *RHOA* with *ROCK* (C) and *ARHGEF1* (□) and *JAK2* (●) with *RHOA* (D) were analysed by Spearman rank correlation and were strongly and highly significantly correlated with each other. Data are shown as Spearman rank coefficient (Rs) and p value. In liver slices of cirrhotic patients, immunofluorescent stainings of α-smooth muscle actin (αSMA), ROCK, pMoesin and DAPI were performed. The coimmunofluorescent stainings showed that ROCK expression and activity, as shown by phosphorylation of its substrate Moesin, is located in αSMA-positive cells of human cirrhotic liver specimen (E).

JAK2 inhibition blunts contractions of activated HSCs in vitro and thereby decreases PP in vivo

The transcription of *Jak2*/*Arhgef1*/*Rock* pathway regulates, at least partly through AT1R, activation of HSCs, as well as their collagen production. However, hepatic fibrosis is only one part of the increased hepatic resistance leading to portal hypertension in liver cirrhosis. The acutely induced contraction of activated HSCs and other contractile cells by vasoconstrictors contributes additionally to increased hepatic resistance in portal hypertension in cirrhosis. In this context, the effect of JAK2 inhibition on HSC contraction was analysed in vitro using 3D stress-relaxed collagen lattice contraction model. These experiments revealed that JAK2 inhibition by AG490 relaxed dose-dependently the contraction of activated HSCs in vitro (figure 5A).

In order to test whether the relaxation of activated HSCs found in vitro after JAK2 inhibition is relevant for modulation of intrahepatic resistance, we performed in situ liver perfusion in CCl_4-intoxicated cirrhotic rats. AG490 dose-dependently decreased hepatic perfusion pressure in cirrhotic livers after vasoconstriction induced by AngII (figure 5B). Interestingly,

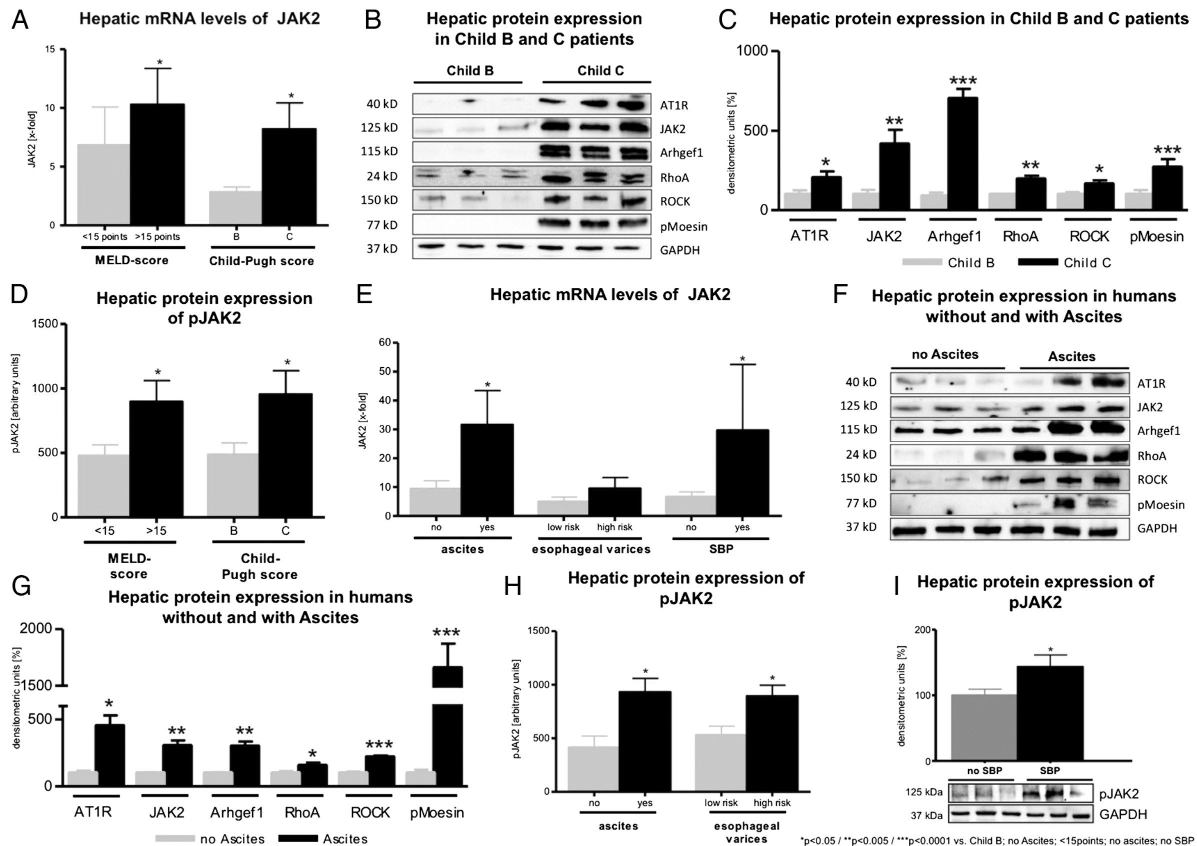

Figure 3 Janus-kinase-2 (JAK2)/RHOA/Rho-kinase (ROCK) pathway is upregulated and overactivated in patients with more severe liver disease and complications of portal hypertension. The hepatic mRNA levels of *JAK2* were highly upregulated in patients with MELD score >15 and Child–Pugh score C compared with patients with MELD <15 and Child–Pugh score B (A). Hepatic protein expression of angiotensin-II-type-I receptor (AT1R), JAK2, ARHGEF1, RHOA, ROCK and pMoesin was increased in liver samples of cirrhotic patients with Child–Pugh score B (n=10 out of 24) and Child–Pugh score C (n=10 out of 20), as shown by representative western blots and (B) and the densitometric quantification (C). The hepatic ROCK activity, as shown by phosphorylation of its substrate moesin at Thr558, was increased in patients with Child–Pugh score C compared with patients with Child–Pugh score B (B and C). The hepatic JAK2 activation (phosphorylation at Tyr1007/1008), measured using ELISA assays, was increased in patients with high MELD score (>15) or with Child–Pugh score C compared with patients with low MELD score (<15) or with Child–Pugh score B (D). Hepatic mRNA levels of *JAK2* were highly upregulated in patients with ascites, with high-risk oesophageal varices or with history of spontaneous bacterial peritonitis (SBP) compared with patients without ascites, low-risk oesophageal varices or without history of SBP (E). Hepatic protein expression of AT1R, JAK2, ARHGEF1, RHOA, ROCK and pMoesin was increased in liver samples of cirrhotic patients with ascites (n=10 out of 32) compared with patients without ascites (n=10 out of 17), as shown by representative western blots (F) and densitometric quantification (G). Hepatic ROCK activity, as shown by phosphorylation of its substrate moesin at Thr558, was increased in patients with ascites compared with patients without ascites (F and G). The hepatic levels of pJAK2 in patients without ascites (n=10) were lower than in patients with ascites (n=10), as shown by ELISA assays (H). In patients with low-risk oesophageal varices, the hepatic expression of pJAK2 was lower than in patients with high-risk oesophageal varices (H). Hepatic protein expression of pJAK2 was significantly increased in liver samples of cirrhotic patients with (n=10) compared with patients without SBP (n=10) (I). The western blot analysis was quantified by densitometry of all experiments with values of the respective control group set to 100 d.u. Relative mRNA was determined by quantitative real time-PCR, normalised to *18S* rRNA and compared with controls (expressed as $2^{-\Delta\Delta Ct}$). Non-parametric Mann–Whitney U statistical test was used to compare between groups and p<0.05 was considered significant. Data are shown as means±SD.

AG490 had no additional effect after AT1R blockade by Losartan indicating the AT1R dependency of the JAK2/ROCK pathway in liver cirrhosis (figure 5B). AG490 led to a less pronounced decrease of the perfusion pressure when livers were precontracted with methoxamine compared with precontraction with AngII (figure 5C).

After having shown the effect of JAK2 inhibition in situ by liver perfusion experiments, we next tested acute haemodynamic effect of JAK2 inhibition in vivo in two different rat models of cirrhotic portal hypertension (BDL and CCl_4). After assessing the baseline haemodynamic situation in these rats by means of microspheres, AG490 was injected. Then, microsphere technique was reapplied to assess the haemodynamic changes by AG490 (figure 5D–F). Injection of AG490 decreased PP in all animals (figure 5D). This was a result of a drop in hepatic resistance (figure 5E). However, JAK2 inhibition also decreased the splanchnic vascular resistance (figure 5F), which led to higher cardiac output (figure 5H). JAK2 inhibition was not accompanied by a change in shunt flow and MAP remained unchanged (figure 5G, I).

Role of JAK2 in extrahepatic vessels

To investigate the mechanisms of AG490 on splachnic vascular resistance, hepatic arteries of human liver transplant donors and recipients and superior mesenteric arteries of cirrhotic rats were used. Interestingly, JAK2 expression and activation as well as downstream effectors were significantly decreased in hepatic arteries of cirrhotic liver transplant recipients compared with

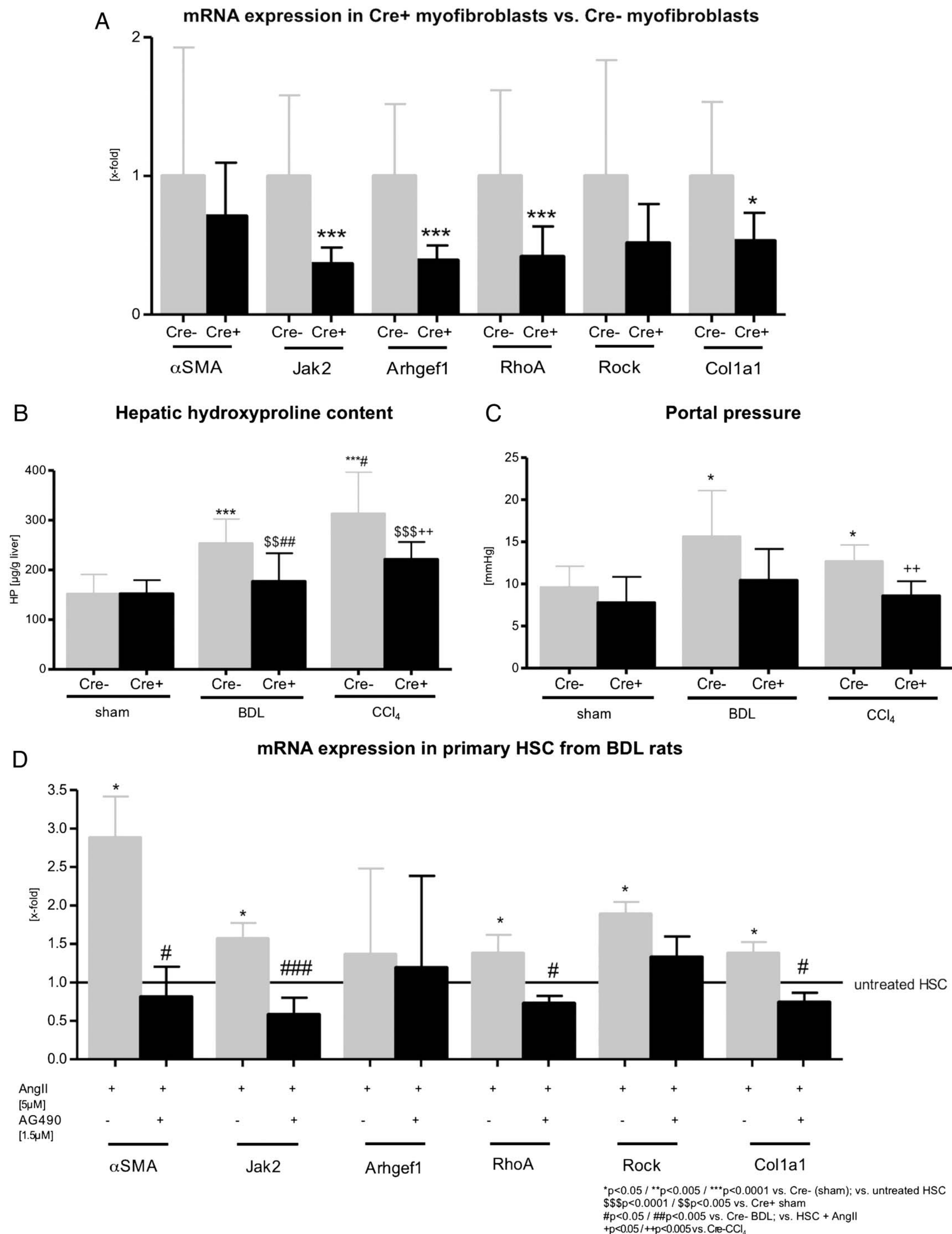

Figure 4 Deficiency of *Janus-kinase-2* (*Jak2*) in myofibroblasts attenuates fibrosis and decreases portal pressure (PP) by downregulation of *Arhgef1/RhoA/Rho-kinase* (*Rock*) pathway. Transcription levels of *αSma*, *Jak2*, *Arhgef1*, *RhoA*, *Rock* and *Col1a1* (collagen-1) were assessed by RT-PCR in myofibroblasts isolated from SM22^{Cre+}-*Jak2*$^{f/f}$ and SM22^{Cre-}-*Jak2*$^{f/f}$ mice (A). Myofibroblasts from SM22^{Cre+}-*Jak2*$^{f/f}$ showed less expression of JAK2/ROCK pathway components than myofibroblasts from SM22^{Cre-}-*Jak2*$^{f/f}$ mice. Hepatic hydroxyproline content in liver samples of SM22^{Cre+}-*Jak2*$^{f/f}$ was significantly lower than SM22^{Cre-}-*Jak2*$^{f/f}$ mice upon liver injury, induced by bile duct ligation (BDL) or carbon tetrachloride (CCl_4), whereas no change was observed in sham-operated animals (B). In vivo invasively measured PP of SM22^{Cre+}-*Jak2*$^{f/f}$ was significantly lower than SM22^{Cre-}-*Jak2*$^{f/f}$ mice upon liver injury, induced by BDL or CCl_4, whereas no significant change was observed in sham-operated animals (C). In vivo activated primary hepatic stellate cells (HSCs) isolated form BDL rats were incubated with angiotensin II (AngII) (5 μM) for 3 days and showed increased transcription of *αSma*, *Arhgef1*, *RhoA*, *Rock* and *Col1a1* (collagen-1) measured by RT-PCR, which was blunted after AG490 (1.5 μM) coincubation (D). For cell culture experiments, duplicates from a minimum of three different animals were used. Relative mRNA was determined by quantitative real time-PCR, normalised to *18S* rRNA as housekeeping gene and compared with controls (expressed as $2^{-\Delta\Delta Ct}$). Non-parametric Mann–Whitney U statistical test was used to compare between two groups (minimum n=6/group), and p<0.05 was considered significant. Data are shown as means±SD. αSMA, α-smooth muscle actin. ### p<0.001 vs HSCs + AngII.

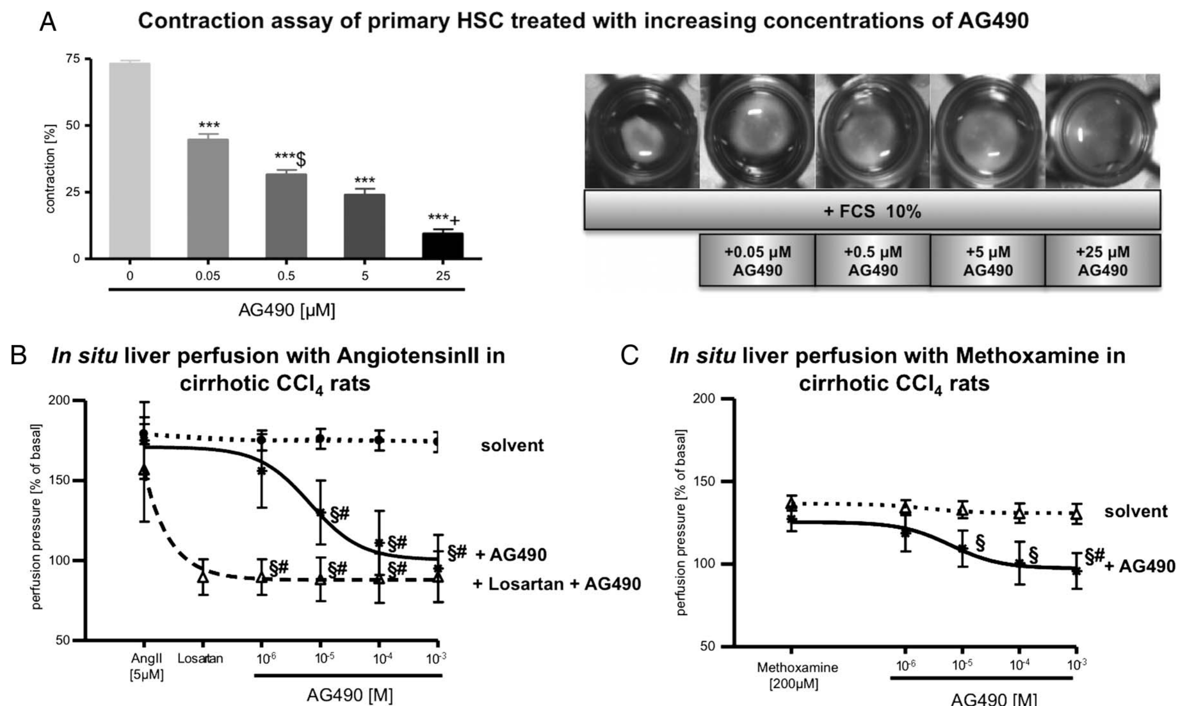

Figure 5 Janus-kinase-2 (JAK2) inhibition blunts contraction of activated hepatic stellate cells (HSCs) and thereby decreases portal pressure (PP) in vivo. Primary isolated rat HSCs were cultured and seeded on a 3D stress-relaxed collagen lattice gel. After addition of serum-free culture medium and different doses of AG490 (0 μM, 0.05 μM, 0.5 μM, 5 μM, 25 μM) to gel lattices with HSCs, cell-mediated contraction was determined by gel diameter and JAK2 inhibition blunted significantly cell contraction (A). Control cell-free gels allowed the quantification of the relative changes in volume in per cent contraction. The contraction of HSCs is expressed as per cent of contraction. For cell culture experiments, duplicates from a minimum of three different animals were used. In situ liver perfusion of cirrhotic carbon tetrachloride (CCl_4)-intoxicated rats analysed the effect of JAK2 inhibition on hepatic resistance (B). The perfusion pressure was measured in CCl_4-intoxicated rat livers without or with increasing doses of AG490 in the perfusate (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) after precontraction with angiotensin II (5 μM). In another set of experiments, Losartan (10 mg/kg) blunted the AG490 effect (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) on perfusion pressure in CCl_4-intoxicated rat livers. In situ liver perfusion of cirrhotic CCl_4-intoxicated rats was additionally performed in rat livers with increasing doses of AG490 (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) after precontraction with the α_1-adrenergic receptor agonist methoxamine (200 μM). AG490 decreased the perfusion pressure dose-dependently compared with rat livers without increasing doses of AG490 in the perfusate (C). The results are shown as percentage of the basal pressure. In vivo invasive measurement of PP (mm Hg) was assessed in cirrhotic bile duct ligation (BDL) and CCl_4 rats before and after AG490 (1 mg/kg) intravenous injection, which showed a significant decrease due to JAK2 inhibition compared with the values before injection (D). The intravenous injection of AG490 (1 mg/kg) decreased the hepatic vascular resistance and the splanchnic vascular resistance (mm Hg min 100 g/mL) in BDL and CCl_4-intoxicated rats compared with the values before injection (E and F). The invasive measurement of mean arterial pressure (mm Hg) did not show any significant change after AG490 (1 mg/kg) injection in BDL and CCl_4-intoxicated rats compared with the values before injection (G). Cardiac output (mL/min/100 g body weight) measured by the microsphere technique was increased in BDL and CCl_4-intoxicated rats after injection of 1 mg/kg AG490 compared with the values before injection (H). The shunt volume (mL/min/100 g body weight) measured by the microsphere technique was not influenced after the injection of 1 mg/kg AG490 compared with values before AG490 injection (I). Non-parametric Mann–Whitney U statistical test was used to compare between two groups (minimum n=6/group), and Wilcoxon test was used for paired groups, respectively, and p<0.05 was considered significant. Data are shown either as means±SD or as paired single animals and the means.

vessels of liver transplant donors (figure 6A–C). Additionally, the JAK2 inhibition using AG490 did not alter JAK2 downstream signalling significantly in vessels of cirrhotic rats (figure 6D–F). To investigate whether AG490 has direct effects on extrahepatic vessels, aortic ring contractions from CCl4 intoxicated rats were performed, which showed dose-dependently a significant relaxation induced by AG490 after precontraction with AngII (figure 6G).

DISCUSSION

Our findings emphasise the fact that intrahepatic JAK2/ROCK activation, both in the experimental setting and in the human disease, is a pivotal pathway associated with liver cirrhosis, especially in advanced disease. It mediates aggravation of portal hypertension and complications. Furthermore, this study demonstrated that acute administration of a JAK2 inhibitor decreased PP and that *Jak2* deletion in αSMA-positive cells blunted fibrogenesis. Thus, JAK2 inhibition may represent a novel therapeutic concept to blunt or interrupt the process towards fibrosis and portal hypertension, especially in advanced cirrhosis, where this pathway is highly activated.

The RAS is critically involved in the development of progressive fibrosis in many organs, including the liver, mainly via AT1R stimulation.[4 10 25–28] In the situation of chronic liver injury, the activation of RAS is a hallmark of progressive liver disease.[2 4 7 29 30] RAS activation not only leads to initiation, perpetuation and augmentation of inflammatory and fibrogenic processes within the liver, with activated HSCs as key players,[5 10] but is also crucially involved in the development and aggravation of portal hypertension associated with formation of ascites and other complications of advanced liver cirrhosis.[8 9 29 30] Recently, our group could confirm the link between AT1R-dependent phosphorylation of JAK2 in activated HSCs and liver fibrosis.[10] Phosphorylation at tyrosine Y1007/1008 is

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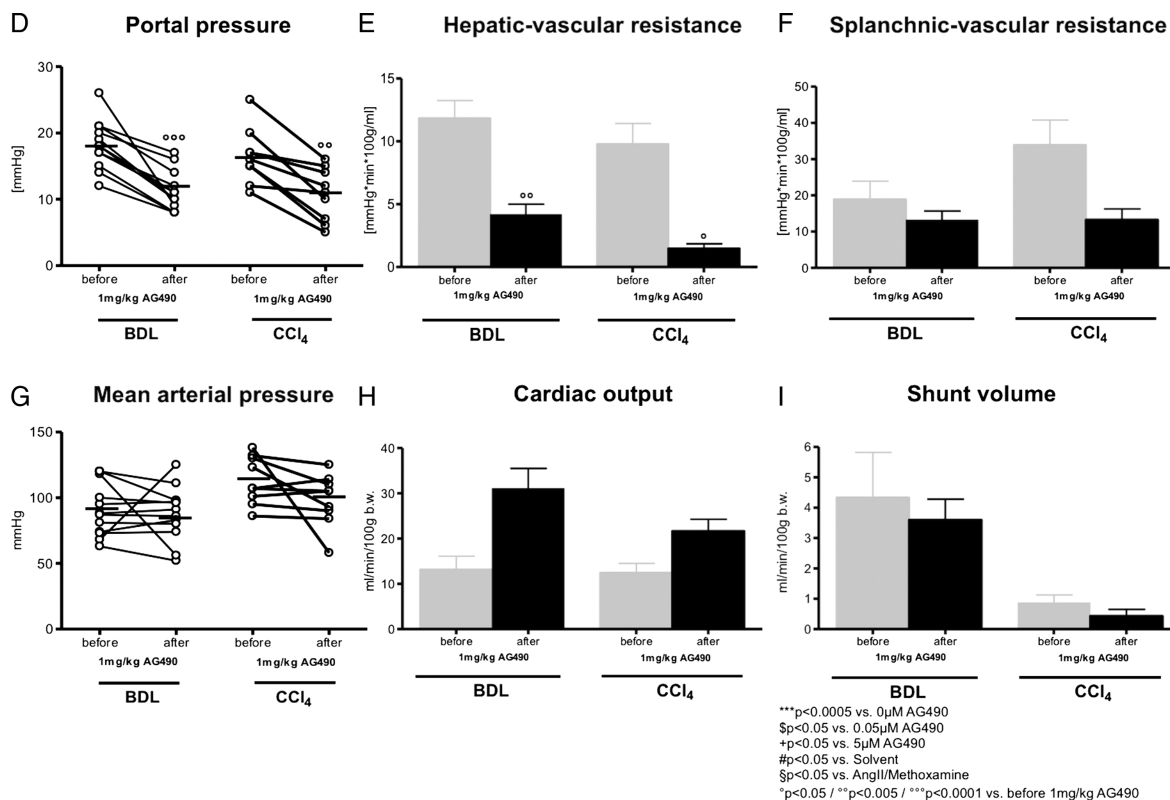

Figure 5 Continued.

necessary for kinase activity, but in contrast to other phosphorylation sites, it has no self-limiting mechanism to inhibit JAK2 activity after receptor stimulation.[31] Phosphorylated JAK2 activates the nucleotide exchange factor for RHOA, ARHGEF1, which then activates the RHOA cascade towards ROCK in HSCs.[10 11] Thus, there are several lines of evidence that stimulation of this pathway via the AT1R leads to activation of HSCs and thereby increased fibrosis, while the inhibition of JAK2 is antifibrotic,[10] which has recently been confirmed by others.[18] The present study assessed the close association of the hepatic RAS pathway—known to be a driver of complications,[4 29 30] more specifically *ACE* and *AT1R*—with the transcription of the *JAK2/ROCK* pathway components in the human liver cirrhosis. It shows that the expression as well as the activation of the AT1R/JAK2/ROCK pathway were significantly upregulated in patients with more severe liver disease, that is, those with a high MELD score and Child–Pugh score, especially when they had complications of portal hypertension. Therefore, the JAK2/ROCK pathway is not only involved in the development of fibrosis, but also in ongoing cirrhosis with complications. Interestingly, this pathway is much more activated in alcoholic cirrhosis compared with chronic viral hepatitis, which further confirms its role and involvement in the fibrogenesis and contraction, rather than in inflammatory processes driven by viral hepatitis. Until now, there are few compounds in debate, which might blunt fibrogenesis and PP in advanced cirrhosis.[3 32] Our novel findings point to another pathway, which could be modulated in this situation, for example, by using JAK2 inhibitors.

In our experimental study, we could link JAK2/ROCK to activated and myofibroblastic HSCs by using in vivo activated myofibroblasts and HSCs taken from fibrotic animals, as well as by using a conditional knock-out model for *Jak2* in αSMA-positive cells. In these animals, even activated myofibroblasts lacked *Jak2*. The *Jak2* deficiency in these cells was associated with a less activated RHOA/ROCK pathway and these animals showed less hepatic fibrogenesis with lower PP in two different models of liver fibrosis. The lower PP in the conditional knock-out model of *Jak2*—or following chronic pharmacological JAK2 inhibition[18]—is not surprising since these animals showed less advanced fibrosis, which is associated with a lower PP through reduction in the 'fixed', 'passive' part of increased intrahepatic vascular resistance. To circumvent this latter factor, we studied the haemodynamic effect of acute pharmacological JAK2 inhibition by use of microspheres. The acute administration of AG490 lowered PP by decreasing intrahepatic vascular resistance in two classically used rodent models of cirrhotic portal hypertension. This effect was strong enough to overcome the increased portal venous inflow due to the concomitant decreased splanchnic vascular resistance after JAK2 inhibition, which was substantiated by data using the aortic ring contraction. Our observation was confirmed by the findings that JAK2 inhibition blunted hepatic resistance of cirrhotic animals in the in situ liver perfusion experiments, and that relaxed dose-dependently activated HSCs in vitro. These rapid haemodynamic effects are plausible since it is known that RHOA/ROCK pathway induces prompt relaxation of contractile cells followed by a fast drop in vascular resistance.[13 15–17 33 34]

The proof of concept in humans is difficult since clinically used JAK2 inhibitors[35 36] have no approval for liver cirrhosis. We had the chance to identify one cirrhotic patient with the indication for ruxolitinib (after bone marrow transplantation for treatment of steroid-refractory graft-versus-host-disease (GvHD)) who underwent transjugular liver biopsy with hepatic venous pressure gradient (HVPG) measurement. In this particular patient, the acute intake of ruxolitinib (5 mg) decreased HVPG from 11 to 6 mm Hg within 30 min after drug intake. At the same time, the portal venous flow increased by 85% from baseline of 12 cm/s. This might be a hint that the hepatic

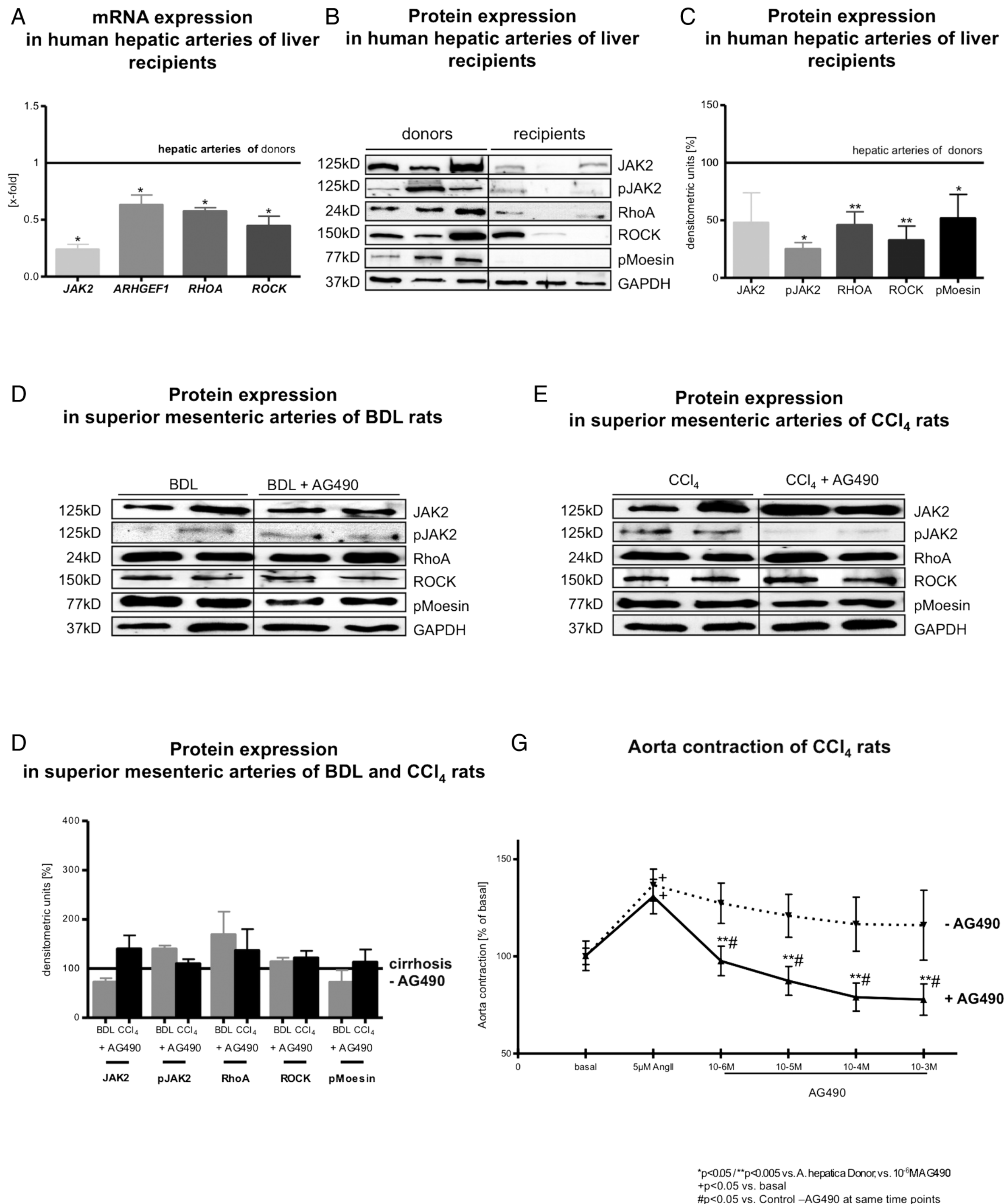

Figure 6 Extrahepatic Janus-kinase-2 (JAK2) inhibition in hepatic arteries of liver donors and recipients in humans and in superior mesenteric arteries and aortic vessels of cirrhotic rats. The hepatic mRNA levels of *JAK2*, *ARHGEF1*, *RHOA* and *ROCK* were measured in hepatic arteries of liver transplant recipients and liver transplant donors using quantitative real time (qRT)-PCR. The mRNA levels of *JAK2*, *ARHGEF1*, *RHOA* and *ROCK* were significantly reduced in hepatic arteries of liver recipients compared with hepatic arteries of liver donors (A). Protein expression of JAK2, pJAK2, RHOA, Rho-kinase (ROCK) and pMoesin was reduced in hepatic arteries of liver recipients compared with hepatic arteries of liver donors, as shown by representative western blots (B) and densitometric quantification (C). Protein expression of JAK2, pJAK2, RHOA, ROCK and pMoesin in superior mesenteric arteries of cirrhotic (bile duct ligation (BDL) and carbon tetrachloride (CCl_4)) rats did not alter significantly compared with superior mesenteric arteries of cirrhotic rats after injection of 1 mg/kg AG490, as shown by representative western blots (D and E) and densitometric quantification (F). In aortic rings of cirrhotic CCl_4-intoxicated rats, a contraction assay with increasing doses of AG490 (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M) was performed and compared with aortic rings without AG490. After precontraction with angiotensin II (AngII) (5 μM), AG490 decreased dose-dependently the contraction of aortic rings compared with aortic rings without AG490 (G). The results are shown as the aorta contraction in per cent of basal contraction. The basal contraction was normalised to 100%. Relative mRNA was determined by quantitative RT-RCR, corrected to *18S* rRNA as housekeeping gene and compared with controls (expressed as $2^{-\Delta\Delta Ct}$). Non-parametric Mann–Whitney U statistical test was used to compare between two groups (minimum n=6/group), and Wilcoxon test was used for paired groups, respectively, and p<0.05 was considered significant. Data are shown either as means±SD or as paired single animals and the means.

resistance dropped in this patient acutely after ruxolitinib intake. Nevertheless, much more information from clinical trials with respect to the situation in cirrhotic patients is required.

In our animal models, the systemic JAK2 inhibition induced concomitant effects in the systemic and splanchnic circulation, namely a vasodilatation. These were compensated by an increase in cardiac output so that the MAP did not drop in the rats. Nevertheless, systemic effects must be carefully observed after JAK2 inhibition, especially in humans.[35 36] Indeed, we could show in this study that JAK2/ARHGEF1/ROCK signalling is defective in human cirrhotic vessels. These findings were complementary to previous studies of our group showing that the ROCK pathway downstream of the AT1 receptor is defective in cirrhotic extrahepatic vessels.[19 20 23] This potential limitation might be bypassed by HSC-specific targeting of JAK2, as recently shown for ROCK inhibition.[15]

In summary, in the present work, we showed that JAK2/ROCK pathway is crucially involved not only in the progression of liver disease, but might also be associated with the development of portal hypertension and related complications. The inhibition of JAK2 decreased activation and contraction of HSCs and PP. This might therefore represent a novel approach to treat portal hypertension in liver cirrhosis, ideally with HSC-specific targeting to reduce intrahepatic resistance.

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Janus-kinase-2 relates directly to portal hypertension and to complications in rodent and human cirrhosis

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